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Development and evaluation of tailored specific real-time RT-PCR assays for detection of foot-and-mouth disease virus serotypes circulating in East Africa

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A B S T R A C T

Rapid, reliable and accurate diagnostic methods provide essential support to programmes that monitor and control foot-and-mouth disease (FMD). While pan-specific molecular tests for FMD virus (FMDV) detection are well established and widely used in endemic and FMD-free countries, current serotyping methods mainly rely either on antigen detection ELISAs or nucleotide sequencing approaches. This report describes the development of a panel of serotype-specific real-time RT-PCR assays (rRT-PCR) tailored to detect FMDV lineages currently circulating in East Africa. These assays target sequences within the VP1-coding region that share high intra-lineage identity, but do not cross-react with FMD viruses from other serotypes that circulate in the region. These serotype-specific assays operate with the same thermal profile as the pan-diagnostic tests making it possible to run them in parallel to produce C_T values comparable to the pan-diagnostic test detecting the 3D-coding region. These assays were evaluated alongside the established pan-specific molecular test using field samples and virus isolates collected from Tanzania, Kenya and Ethiopia that had been previously characterised by nucleotide sequencing. Samples (n = 71) representing serotype A (topotype AFRICA, lineage G-I), serotype O (topotypes EA-2 and EA-4), serotype SAT 1 (topotype I (NWZ)) and serotype SAT2 (topotype IV) were correctly identified with these rRT-PCR assays. Furthermore, FMDV RNA from samples that did not contain infectious virus could still be serotyped using these assays. These serotype-specific real-time RT-PCR assays can detect and characterise FMDVs currently circulating in East Africa and hence improve disease control in this region.

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease affecting both wild and domesticated cloven-hooved animals. Although FMD generally has a low mortality rate, it affects a large number of animals of multiple species leading to loss or reduction in livestock production in endemic regions in Asia and Africa. It is estimated that collectively three-quarters of the world's livestock population is concentrated in FMD endemic areas (Knight-Jones and Rushton, 2013) leading to an enormous source of inefficiency and waste in food production especially in the context of a growing demand for livestock products. In addition, outbreaks of the disease can spread into countries normally free of FMD posing significant costs to livestock industries.

FMD is caused by FMD virus (FMDV), belonging to genus *Aphthovirus* within the family *Picornaviridae* (Knowles et al., 2012). The non-enveloped virus particle encloses a single-stranded positive-sense RNA genome of approximately 8.3 kilobases. Seven immunologically distinct serotypes of the virus exist: O, A, C, SAT (Southern African Territories) 1, SAT 2, SAT 3 and Asia 1; each

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containing multiple variants (topotypes) often restricted to specific geographical locations. Global distribution of FMDV serotypes is uneven. Currently, serotypes O and A have the widest distribution and are reported in most endemic virus pools.

The surface exposed capsid protein, VP1, plays an important role in antigenic and phylogenetic characterisation of FMDV as it contains major immunogenic sites, including amino acid residues within the G-H loop and the C-terminus (Baranowski et al., 2000; Jackson et al., 2002). The VP1-coding nucleotide sequence varies between FMDV serotypes. Further classification of viruses into topotypes and lineages is also possible based on phylogenetic analysis of the sequence of the VP1-coding region (Knowles and Samuel, 2003; Samuel and Knowles, 2001). The genetic relationship of the VP1 nucleotide sequence between different strains is commonly applied for tracing the origin and movement of outbreak viruses (Abdul-Hamid et al., 2011; Di Nardo et al., 2014; Knowles et al., 2014).

The established methods for FMD control, such as vaccination and movement restrictions (Sumption et al., 2012), are underpinned by rapid and accurate diagnosis of clinical cases of the disease. Currently, pan-specific real-time reverse transcription polymerase chain reaction (rRT-PCR) assays for FMDV detection have been described (Callahan et al., 2002; Moniwa et al., 2007; Reid et al., 2002, 2009). These amplify highly conserved RNA sequences within the 5′-untranslated region (UTR) or the RNA polymerase (3D-coding region), respectively. As these genome regions are highly conserved for all seven serotypes, FMDV can be detected with high diagnostic sensitivity and specificity. However, rapid identification of the serotype of an outbreak virus is necessary for vaccine selection, especially in endemic countries. This is normally achieved by antigen detection ELISA and/or VP1 sequencing (Ferris and Dawson, 1988; Knowles et al., in press). With both methods often requiring virus isolation and propagation for increased sensitivity, the time for serotype identification and therefore for implementation of effective vaccination can be prolonged.

As the VP1 coding region nucleotide sequence varies according to the serotype of FMDV, typing can be achieved by serotype-specific detection of the VP1 coding region, ideally, without the need for virus propagation. Conventional RT-PCR assays targeting the VP1 coding region have been described (Alexandersen et al., 2000; Callens and de Clercq, 1997; Vangrysperre and de Clercq, 1996) but due to the high genetic diversity within the target region these have relatively poor sensitivity and specificity (Reid et al., 2001). A microarray-based system for typing FMDV has also been described (Baxi et al., 2006) but this can be lengthy and costly and also requires use of specialised equipment. Sequence data analysis of the VP1 coding region indicates that, due to the extensive intra-serotype variation of the VP1 coding nucleotide sequences, development of reliable serotype specific RT-PCR assays might be difficult to achieve on a global basis (Reid et al., 1999).

As FMDV circulation often occurs in regional reservoirs (Paton et al., 2009), topotypes and lineages of FMDV specific to different geographical areas have evolved. These can be identified and characterised based on phylogenetic analysis of the VP1 coding sequences. Exploiting the fact that genetic variability between strains within lineages is reduced in comparison to the nucleotide variability within serotypes, RT-PCR assays tailored to FMDV lineages and therefore geographic areas can be developed. This idea has been applied in conventional RT-PCR systems to distinguish between strains of FMDV belonging to different serotypes circulating in India (Giridharan et al., 2005). This system was further developed and applied to serotype viruses circulating in the Middle East (Reid et al., 2014), West Eurasia (Jamal and Belsham, 2015) as well as for the detection of the SAT 2 serotype (topotype VII) in Egypt (Ahmed et al., 2012) and the serotype O viruses in North Africa belonging to Ind-2001d lineage (Knowles et al., 2014).

Table 1
List of primers and probes for FMDV type-specific assays in East Africa.

OLIGO NAME	NUCLEOTIDE SEQUENCE (5′ → 3′)
FMDV/A/FP	GCCACRACCATCCACGA
FMDV/A/RP	GAAGGGCCCAGGGTTGGACTC
FMDV/A/P	FAM-CTCGTGCGMATGAARCGGGC-BHQ1
FMDV/O/FP	CCTCCTTCAAYTACGGTG
FMDV/O/RP	GCCACAATCTTYTGTTTGTG
FMDV/O/P	FAM-CCCTCTTCATGCGGTARAGCAG-BHQ1
FMDV/SAT1/FP	CTYGACCGGTTCACYCTG
FMDV/SAT1/RP	CCGAGAAGTAGTACGTRGC
FMDV/SAT1/P	FAM-CAGGAYTGCGCCCACCA-BHQ1
FMDV/SAT2/FP	CRATCCGCGGTGAYCG
FMDV/SAT2/RP	CGCTTCATYCTGTAGTARACGTC
FMDV/SAT2/P	FAM-TTTGGACAYGTGACCGCCG-BHQ1

The names of the primers and probes indicate the type-specificity of the assays. FP, RP or P stand for "forward primer", "reverse primer" or "probe" respectively; R = A or G, Y = C or T and M = A or C.

This study describes the development of a set of typing rRT-PCR assays tailored to detect FMDV viruses currently circulating in East Africa, with a particular focus on Tanzania, Kenya and Uganda.

2. Materials and methods

2.1. Primers and probe design

The design of primers and probes was based on nucleotide sequences encoding VP1 originating from East Africa obtained from GenBank (http://www.ncbi.nlm.nih.gov/).

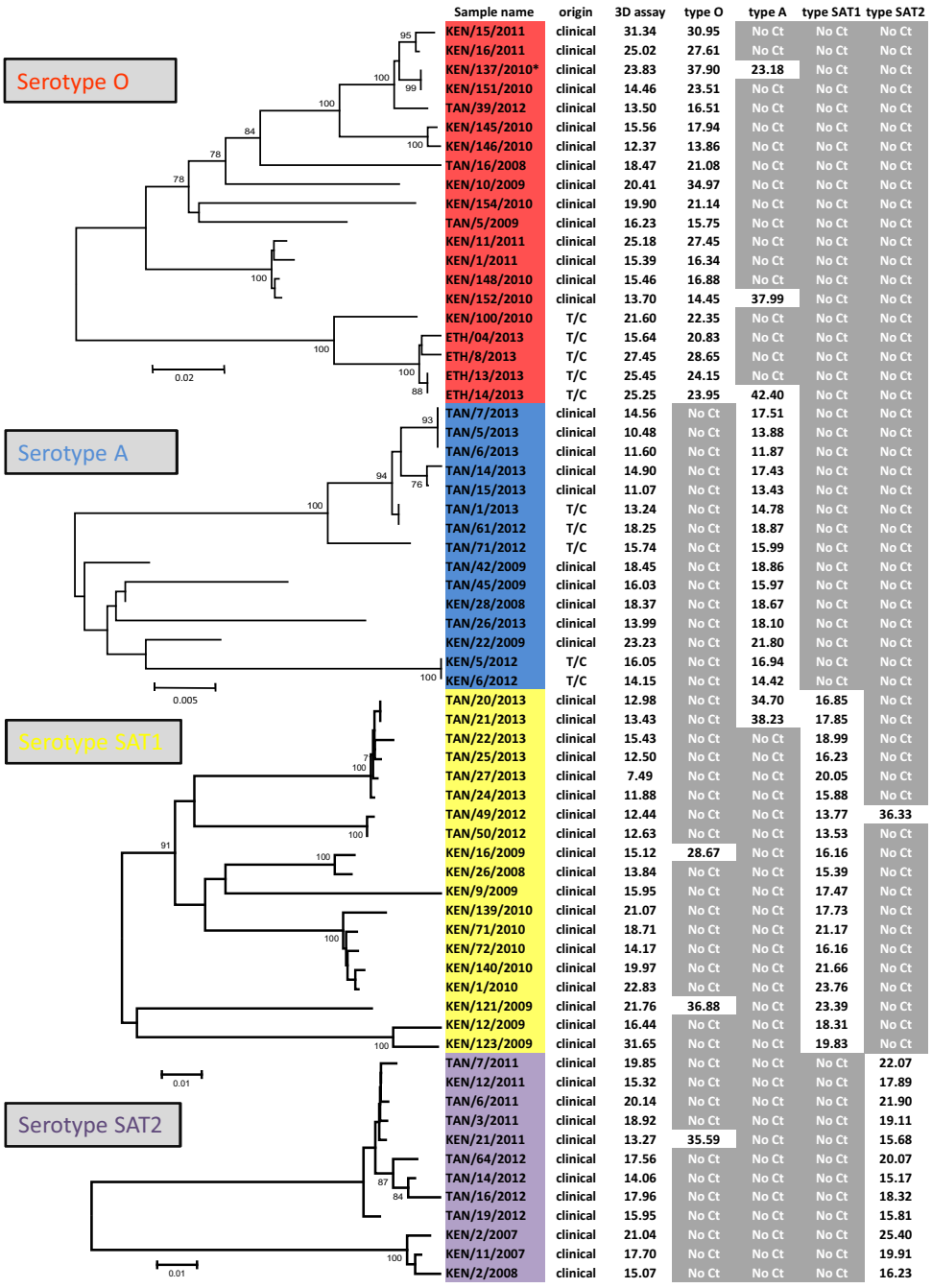
The VP1 coding region sequences were aligned using BioEdit (Hall, 1999) and analysed collectively and separately for serotypes A, O, SAT 1 and SAT 2. Following the analyses, unique regions of the VP1 coding sequence were identified as targets for primers and probes for each serotype, in accordance with TaqMan specifications. However, to achieve satisfactory results in the type A assay, a modified NK72 primer of broad specificity that anneals to the nucleotide sequence at the junction of 2A and 2B coding region (Knowles et al., in press), was used as the reverse primer. Each oligonucleotide (oligo) set was evaluated *in silico* to ensure diagnostic specificity between the different lineages within the serotypes without a compromise on cross-reactivity between serotypes.

All oligonucleotides were synthesised by Sigma-Aldrich (USA) and all probes were labelled with BHQ-1 (Black Hole Quencher-1) and FAM at their 3′ and 5′ termini, respectively.

At least two candidate oligo sets (primers and probe) were designed for each of the assays and evaluated in multiple possible combinations for detection of the homologous serotype. The best performing oligo was selected for each of the respective assays. Table 1 lists the sequences of the best performing oligo sets.

2.2. Virus isolates

A panel of FMDV clinical samples (n = 61) and virus isolates (n = 10) was selected from the sample repository held at the World Reference Laboratory for FMD (WRLFMD) (The Pirbright Institute, UK) and contemporary diagnostic submissions from East Africa (Fig. 1). These included FMDV samples previously characterised as serotype A, topotype AFRICA, lineage G-I; serotype O, topotype EA-2 and EA-4; serotype SAT 1, topotype I (NWZ) and serotype SAT 2, topotype IV. Virus was isolated from these samples and tested with antigen ELISA (Ag-ELISA) to establish the FMDV serotype (Ferris and Dawson, 1988). Samples that originated from East Africa, from which FMDV RNA was detected by rRT-PCR (Callahan et al., 2002), but neither virus isolation nor Ag-ELISA were positive (TAN/9/2013, TAN/16/2013,TAN/18/2013, TAN/23/2013 and TAN/28/2013) were also included in the panel.



Sample name	origin	3D assay	type O	type A	type SAT1	type SAT2
KEN/15/2011	clinical	31.34	30.95	No Ct	No Ct	No Ct
KEN/16/2011	clinical	25.02	27.61	No Ct	No Ct	No Ct
KEN/137/2010*	clinical	23.83	37.90	23.18	No Ct	No Ct
KEN/151/2010	clinical	14.46	23.51	No Ct	No Ct	No Ct
TAN/39/2012	clinical	13.50	16.51	No Ct	No Ct	No Ct
KEN/145/2010	clinical	15.56	17.94	No Ct	No Ct	No Ct
KEN/146/2010	clinical	12.37	13.86	No Ct	No Ct	No Ct
TAN/16/2008	clinical	18.47	21.08	No Ct	No Ct	No Ct
KEN/10/2009	clinical	20.41	34.97	No Ct	No Ct	No Ct
KEN/154/2010	clinical	19.90	21.14	No Ct	No Ct	No Ct
TAN/5/2009	clinical	16.23	15.75	No Ct	No Ct	No Ct
KEN/11/2011	clinical	25.18	27.45	No Ct	No Ct	No Ct
KEN/1/2011	clinical	15.39	16.34	No Ct	No Ct	No Ct
KEN/148/2010	clinical	15.46	16.88	No Ct	No Ct	No Ct
KEN/152/2010	clinical	13.70	14.45	37.99	No Ct	No Ct
KEN/100/2010	T/C	21.60	22.35	No Ct	No Ct	No Ct
ETH/04/2013	T/C	15.64	20.83	No Ct	No Ct	No Ct
ETH/8/2013	T/C	27.45	28.65	No Ct	No Ct	No Ct
ETH/13/2013	T/C	25.45	24.15	No Ct	No Ct	No Ct
ETH/14/2013	T/C	25.25	23.95	42.40	No Ct	No Ct
TAN/7/2013	clinical	14.56	No Ct	17.51	No Ct	No Ct
TAN/5/2013	clinical	10.48	No Ct	13.88	No Ct	No Ct
TAN/6/2013	clinical	11.60	No Ct	11.87	No Ct	No Ct
TAN/14/2013	clinical	14.90	No Ct	17.43	No Ct	No Ct
TAN/15/2013	clinical	11.07	No Ct	13.43	No Ct	No Ct
TAN/1/2013	T/C	13.24	No Ct	14.78	No Ct	No Ct
TAN/61/2012	T/C	18.25	No Ct	18.87	No Ct	No Ct
TAN/71/2012	T/C	15.74	No Ct	15.99	No Ct	No Ct
TAN/42/2009	clinical	18.45	No Ct	18.86	No Ct	No Ct
TAN/45/2009	clinical	16.03	No Ct	15.97	No Ct	No Ct
KEN/28/2008	clinical	18.37	No Ct	18.67	No Ct	No Ct
TAN/26/2013	clinical	13.99	No Ct	18.10	No Ct	No Ct
KEN/22/2009	clinical	23.23	No Ct	21.80	No Ct	No Ct
KEN/5/2012	T/C	16.05	No Ct	16.94	No Ct	No Ct
KEN/6/2012	T/C	14.15	No Ct	14.42	No Ct	No Ct
TAN/20/2013	clinical	12.98	No Ct	34.70	16.85	No Ct
TAN/21/2013	clinical	13.43	No Ct	38.23	17.85	No Ct
TAN/22/2013	clinical	15.43	No Ct	No Ct	18.99	No Ct
TAN/25/2013	clinical	12.50	No Ct	No Ct	16.23	No Ct
TAN/27/2013	clinical	7.49	No Ct	No Ct	20.05	No Ct
TAN/24/2013	clinical	11.88	No Ct	No Ct	15.88	No Ct
TAN/49/2012	clinical	12.44	No Ct	No Ct	13.77	36.33
TAN/50/2012	clinical	12.63	No Ct	No Ct	13.53	No Ct
KEN/16/2009	clinical	15.12	28.67	No Ct	16.16	No Ct
KEN/26/2008	clinical	13.84	No Ct	No Ct	15.39	No Ct
KEN/9/2009	clinical	15.95	No Ct	No Ct	17.47	No Ct
KEN/139/2010	clinical	21.07	No Ct	No Ct	17.73	No Ct
KEN/71/2010	clinical	18.71	No Ct	No Ct	21.17	No Ct
KEN/72/2010	clinical	14.17	No Ct	No Ct	16.16	No Ct
KEN/140/2010	clinical	19.97	No Ct	No Ct	21.66	No Ct
KEN/1/2010	clinical	22.83	No Ct	No Ct	23.76	No Ct
KEN/121/2009	clinical	21.76	36.88	No Ct	23.39	No Ct
KEN/12/2009	clinical	16.44	No Ct	No Ct	18.31	No Ct
KEN/123/2009	clinical	31.65	No Ct	No Ct	19.83	No Ct
TAN/7/2011	clinical	19.85	No Ct	No Ct	No Ct	22.07
KEN/12/2011	clinical	15.32	No Ct	No Ct	No Ct	17.89
TAN/6/2011	clinical	20.14	No Ct	No Ct	No Ct	21.90
TAN/3/2011	clinical	18.92	No Ct	No Ct	No Ct	19.11
KEN/21/2011	clinical	13.27	35.59	No Ct	No Ct	15.68
TAN/64/2012	clinical	17.56	No Ct	No Ct	No Ct	20.07
TAN/14/2012	clinical	14.06	No Ct	No Ct	No Ct	15.17
TAN/16/2012	clinical	17.96	No Ct	No Ct	No Ct	18.32
TAN/19/2012	clinical	15.95	No Ct	No Ct	No Ct	15.81
KEN/2/2007	clinical	21.04	No Ct	No Ct	No Ct	25.40
KEN/11/2007	clinical	17.70	No Ct	No Ct	No Ct	19.91
KEN/2/2008	clinical	15.07	No Ct	No Ct	No Ct	16.23

Fig. 1. Details of the FMDV positive samples by virus isolation used in this study. Phylogenetic comparisons are based on the analysis of VP1 coding sequence. T/C stands for virus propagated in tissue culture.
*Sample KEN/137/2010 sequence data was obtained from virus propagated in tissue culture (BTy2) (serotype O) while rRT-PCR results (serotype A) were obtained directly from clinical samples.

Additionally 27 field samples collected in Tanzania were tested in the Tanzania Veterinary Laboratory Agency (TVLA) to confirm the diagnostic performance of the developed assays in an African setting.

2.3. RNA sample preparation

Nucleic acid was extracted either using an automated extraction platform, MagNA Pure (Roche) as previously described (Reid et al., 2003) or manually using a QIAamp Viral RNA MiniKit (Qiagen) according to the manufacturer's instructions. Each of the isolates was handled with care to avoid any cross-contamination. The viral RNA was stored at −70 °C until used in the experiments.

2.4. RT-PCR

The respective primers and probe sets were designed to perform under the same protocol and amplification conditions as those described earlier for the pan-FMDV assays (Shaw et al., 2007) with the view that pan-specific and tailored assays could be performed in parallel on the same reaction plate. FMDV samples were also tested using a pan-specific assay detecting the 3D-coding region (Callahan et al., 2002) to validate the results. All reactions were performed in

duplicate, set up manually and amplified in a Stratagene Mx3005P thermal cycler.

The amplification efficiency of each of the respective typing assays was estimated on the basis of a 10-fold dilution of a representative sample for each serotype (serotype O: ETH/4/2013; serotype A: TAN/45/2009; serotype SAT 1: KEN/136/2010; serotype SAT 2: KEN/21/2011). The efficiency was calculated by the following formula: $E\% = 10^{-1/slope} \times 100$.

2.5. Sequencing

The VP1 sequences were generated using the standard method and primers (Knowles et al., in press) by 3730 DNA Analyzer (ABI), according to the manufacturer's instructions and assembled using SeqMan Pro 13 Software (DNAStar Inc., USA). Sequence data were aligned using CLUSTAL W v1.83 software (Thompson et al., 1994) and phylogenetic trees constructed by Neighbor-Joining method. The evolutionary distances were computed using the Kimura 2-parameter method in MEGA version 6.06 software (1000 bootstrap replicates) (Tamura et al., 2013).

3. Results

3.1. Diagnostic sensitivity and specificity of type-specific rRT-PCR assays

A panel of 68 RNA samples representing FMD viruses of serotypes O, A, SAT 1 and SAT 2 circulating in Kenya, Tanzania and Ethiopia collected between 2007 and 2013 was tested using the type-specific rRT-PCR assays. Results for duplicate RNA samples were directly compared to those of the 3D rRT-PCR assay (Callahan et al., 2002). All FMDV RNA samples were detected by the assay targeting the 3D-coding region and the serotype of these positive samples was determined using the corresponding type-specific rRT-PCR assay, identifying virus type with comparable C_T values in most cases (Fig. 1). Mean C_T values for the 3D assay and serotype-specific assays (O, A, SAT 1 and SAT 2) were calculated for the isolates tested showing that, on average, isolates were detected earlier with the pan-specific 3D assay with a reduction of 2.9, 1.1, 1.7 and 1·7 C_T values, respectively. The virus type identified by the type-specific real-time RT-PCR assay was confirmed from VP1 nucleotide sequence data (data not shown).

In nine RNA samples, a positive signal was produced with two different serotype-specific assays. In seven of these, the serotype of the virus was correctly identified by the assay which produced a C_T value similar to the C_T value of the 3D targeted assay, while the second assay produced a C_T value >12.44 higher in the panel of samples tested. However, in sample KEN/137/2010, which was characterised by VP1 sequencing and Ag-ELISA (WRLFMD report: http://www.wrlfmd.org/fmd_genotyping/2011/WRLFMD-2011-00008%20O%20Kenya%202010-2011.pdf) as serotype O, RNA of FMDV serotypes A and O was detected. In the type A-specific rRT-PCR assays the C_T value (23.18) was comparable to the 3D pan-specific assay (23.83), while the serotype O signal was weaker (C_T value of 37.90). The A type-specific rRT-PCR assay amplicon was sequenced, confirming the presence of the serotype A RNA in this sample. The amplicon sequence was compared to other known contemporary serotype A sequences from the geographical area showing that this virus in KEN/137/2010 has a novel sequence not previously handled at the Pirbright Laboratory (data not shown).

In addition to samples that had been previously characterised by Ag-ELISA and/or VP1 sequencing, the virus serotype was identified in five samples in which the FMDV genome was detected with the pan-specific assay targeting the 3D region but which were negative by virus isolation methods. FMDV type A-specific assay produced a positive signal in three of the samples tested (TAN/9/2013, TAN/16/2013 and TAN/18/2013) while SAT 1 virus template was detected with the SAT 1-specific assay in two other samples (TAN/23/2013 and TAN/28/2013). The C_T values in each case were comparable to the C_T value of the 3D targeting assay.

3.2. Analytical sensitivity

The comparative analytical sensitivity of the type-specific assays was evaluated based on ten-fold dilution series of homologous viral RNA performed in duplicated in direct comparison to the pan-specific assay. Dilutions of homologous viral RNA were detected at similar C_T values for each of the type-specific assays in comparison to the pan-specific assay. However, the A and SAT 2-specific assays detected seven serial dilution of the RNA template, while type O- and SAT 1-specific assays yielded positive results with six dilutions, one fewer than the pan-specific assay (Fig. 2).

The efficiency of the respective type-specific assays varied between 92.9 and 101.3% and was comparable to that of the assay detecting the 3D coding region (efficiency between 89.2 and 100.2%) (Fig. 2), confirming similar robustness of the type-specific assay to that of the 3D specific assay.

3.3. Evaluation of assays in East Africa

The performance of the type-specific assays was further evaluated alongside the pan-specific 3D assay (Callahan et al., 2002) in the Tanzania Veterinary Laboratory Agency where an additional panel of 27 clinical samples collected in Tanzania between 2008 and 2013, two FMDV positive controls and a negative control were tested. All samples, apart from the negative control, were detected with the pan-specific assay targeting the 3D region with C_T values ranging from 10.19 to 37.17. The type-specific C_T values ranged from 9.72 to 31.89 with all but the negative control and two samples (#29 and #179) which produced a weak 3D signal (C_T 37.17 and 34.32, respectively) (Table 2). The type-specific signal gave a clear indication of the serotype for the majority of samples, however, a positive signal was recorded in sample #374 with two type-specific assays – A and SAT 1, possibly indicating a mix infection. A high C_T value (31.90–39.02) was recorded with a number of samples indicating detection of a low level of viral RNA.

4. Discussion

The availability of rapid, reliable and accurate diagnostic methods is important for effective control and surveillance strategies for FMD. Methods for the detection of FMDV such as virus isolation, conventional and rRT-PCR are widely used while the repertoire of serotyping methods (e.g. ELISA) is more limited (OIE, 2012). The serotyping antigen ELISA often relies on initial virus isolation and propagation and requires access to standard biological reagents (Ferris and Dawson, 1988). These factors may extend the period for implementation of control strategies especially in endemic countries and make surveillance strategies labour- and cost-intensive.

The FMDV genome region encoding the capsid proteins, particularly the VP1 coding sequence, varies according to serotype. However, the high level of intra-serotype nucleotide variability makes it difficult to design broadly reacting molecular tests that can detect all representatives of a single FMDV serotype that are circulating in different geographical regions (Reid et al., 2014). Instead, a number of molecular assays tailored to a specific lineage found in more restricted geographical locations have been successfully developed (Ahmed et al., 2012; Jamal and Belsham, 2015; Knowles et al., 2014; Reid et al., 2014). This study also employed this approach and describes the design and validation of FMDV type-specific assays tailored for the FMD viruses from East Africa.

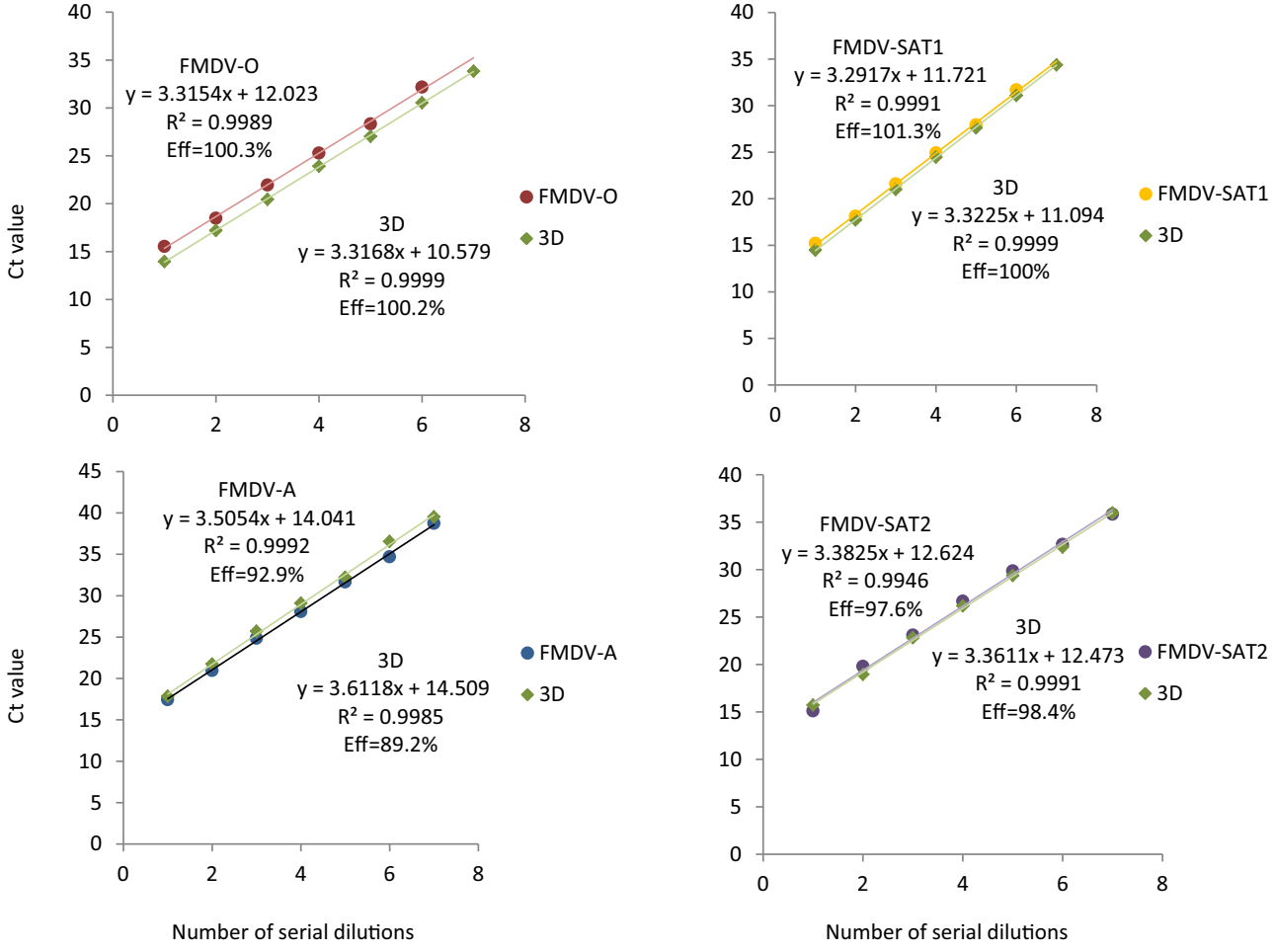


Fig. 2. Comparison of detection of serial diluted viral RNA in pan-specific (3D-specific) and individual type-specific assays.

FMDV circulation often occurs within regional reservoirs where strains specific to the region evolve (Paton et al., 2009). Thus, mainly four serotypes of FMDV (O, A, SAT 1 and SAT 2) have been described to circulate in East Africa. Within each serotype, a restricted number of topotypes/lineages have been identified, namely: topotype EA-2 and EA-4 in FMDV O, topotype AFRICA, lineage G-I in FMDV A, topotype I (NWZ) in FMDV SAT1 and topotype IV in FMDV SAT2 (Kasambula et al., 2012; Kasanga et al., 2015; Namatovu et al., 2015; Wekesa et al., 2014, 2015a,b). In this study, a set of four serotype-specific TaqMan technology based assays was designed, tailored to detect the various FMDV serotypes currently circulating in East Africa. This was achieved by targeting the serotype-specific VP1 coding sequence in most cases. However, the reverse primer in the FMDV-A specific assay was designed to anneal to the more conserved 2A/2B coding region of the genome, due to high heterogeneity within the VP1 coding region of the analysed strains.

As intended, the assays were shown to specifically identify the homologous viruses in all assays with the C_T values similar to those of the pan-specific test detecting the 3D coding region, identifying the serotype of the sample. In addition to studies undertaken in Europe (at The Pirbright Institute), validation experiments were also conducted at the Tanzania Veterinary Laboratory Agency showing that rapid and reliable identification of the virus type can be achieved in an endemic setting. In some samples, however, a positive signal was recorded in more than one of the serotype-specific RT-PCR assays. This could be indicative of a mixed infection, a laboratory contamination or poor specificity of the developed tests. However, *in silico* analyses of the respective oligo-template annealing regions indicated high specificity of these assays. In addition, the assay's specificity was investigated by sequencing of the amplicons and the presence of VP1 templates as identified by the developed rRT-PCR assays was confirmed in each case.

The analysis of the results obtained from testing different passages of the KEN/137/2010 sample give insight into the veracity of the assays developed. FMDV from this clinical sample was isolated and passaged twice in BTy cells and the serotype was identified as FMDV-O in Ag-ELISA by the WRLFMD laboratory (The Pirbright Institute). The VP1 sequence of the same serotype was also obtained on the virus isolate confirming the results of serological typing. However, when RNA extracted directly from the clinical material was tested with the developed typing rRT-PCR assays, the presence of two serotypes was identified. The FMDV-O specific assay produced a late signal (high C_T value) (indicating a low level of the serotype O genetic material) and the FMDV-A specific assay generated a lower C_T value comparable to the pan-specific assay, indicating that the predominant serotype in the initial sample was FMDV-A. The rRT-PCR amplicon of the FMDV-A specific assay was subsequently sequenced and compared to FMDV-A sequences available in the WRLFMD database showing it as a novel sequence. These results lead to a conclusion that the FMDV-O component of the virus present in the clinical samples may have become dominant during subsequent cell passages outcompeting the predominant A virus which was present in the clinical sample. Alternatively, laboratory contamination could have played a role.

Table 2
Validation of the serotype specific assays in laboratory settings in Tanzania.

Sample id	Village/Town	Region	Collection date	rRT-PCR Ct value with primer/probe sets					
				3D	O	A	SAT1	SAT2	Result
3	Wasela	Tanga	12/08/2008	23.97	22.74	No Ct	No Ct	No Ct	O
4	Ngara	Kagera	02/12/2010	29.48	29.45	No Ct	No Ct	No Ct	O
8	Mabuki	Mwanza	16/12/2010	22.07	31.59	No Ct	No Ct	No Ct	O
19	Sumbawanga	Rukwa	16/10/2010	24.57	25.71	No Ct	No Ct	39.02	O
37	Municipal Tabora	Tabora	21/09/2010	19.99	22.12	No Ct	No Ct	No Ct	O
45	Cheyo	Tabora	13/05/2009	23.66	30.34	33.56	No Ct	No Ct	O
48	Nzega	Tabora	02/09/2010	27.65	31.89	No Ct	No Ct	No Ct	O
61	Sumbawanga	Rukwa	2010	27.37	27.73	No Ct	No Ct	No Ct	O
116	Unguja	Zanzibar	23/10/2010	25.18	28.66	No Ct	No Ct	34.80	O
142	Mabuki	Mwanza	16/12/2010	15.78	24.63	No Ct	No Ct	37.94	O
319	Uyole	Mbeya	31/05/2012	24.47	25.02	36.85	No Ct	38.21	O
321	Uyole	Mbeya	31/05/2012	20.14	21.87	No Ct	No Ct	38.10	O
376	Muleba	Kagera	04/04/2013	22.32	24.29	No Ct	No Ct	No Ct	O
379	Muleba	Kagera	2013	23.58	No Ct	25.18	No Ct	No Ct	A
370	Misenyi	Kagera	04/04/2013	19.48	38.26	20.39	No Ct	No Ct	A
374	Karagwe	Kagera	2013	21.87	38.33	22.89	25.97	No Ct	A, SAT 1
355	Kingolowila	Morogoro	31/08/2012	20.85	35.87	No Ct	16.61	38.04	SAT 1
357	Mkambara	Morogoro	31/08/2012	22.37	34.21	34.81	20.77	No Ct	SAT 1
360	Kimara Ilala	Dar-es-salaam	15/12/2011	23.57	35.76	No Ct	20.47	37.50	SAT 1
361	Kimara Ilala	Dar-es-salaam	21/09/2011	14.47	37.65	No Ct	9.72	No Ct	SAT 1
368	Muleba	Kagera	2013	27.86	No Ct	31.90	24.67	No Ct	SAT 1
359	Kimara Ilala	Dar-es-Salaam	02/10/2012	15.65	39.42	No Ct	11.95	37.92	SAT 1
68	Masasi	Mtwara	19/12/2011	21.37	No Ct	No Ct	No Ct	23.24	SAT 2
77	Mazimbu	Morogoro	19/03/2012	24.23	36.42	37.20	32.90	24.92	SAT 2
126	Mavovo	Tanga	21/09/2011	22.84	No Ct	No Ct	No Ct	23.04	SAT 2
29	Kasulu	Kigoma	13/11/2010	37.17	No Ct	No Ct	No Ct	No Ct	NONE
179	Nyegezi	Mwanza	2011	34.32	No Ct	No Ct	No Ct	No Ct	NONE
PC1				10.19	10.30	No Ct	No Ct	No Ct	O
PC2				17.99	No Ct	23.62	No Ct	No Ct	A
NC	Mbagala	Mwanza	05/12/2011	No Ct	No Ct	No Ct	No Ct	No Ct	NONE

PC-positive control; NC- negative control

PC—positive control; NC—negative control.

These data also show that the rRT-PCR assays can be used on specimens where virus isolation is not available or not possible. This feature gives the ability to obtain epidemiological information even from samples not suitable for serotyping by Ag-ELISA. The ability to establish the type of the virus by selective genome amplification makes this set of type-specific assays suitable for further applications such as adaptation for mobile diagnostic platforms (Howson et al., 2015; Madi et al., 2012) and/or validation for use in combination with nucleic acid recovered from lateral-flow devices (Fowler et al., 2014). Furthermore, the type-specific assays operate with the same thermal profile as the pan-diagnostic tests making it possible to multiplex diagnostic and serotyping tests further enhancing the potential for these assays.

These findings highlight the validity of the East African serotyping RT-PCR assay in a wide range of applications including testing for mixed infection or lab contamination of viral RNA samples designed for further downstream applications such as production of diagnostic sera. FMDV is a rapidly evolving virus and the developed assays target the most variable region of its genome. It is therefore possible that, with time, new lineages will evolve or strains are discovered with significant changes within the oligo annealing sites, prohibiting normal function of these assays. It is therefore recommended that such assays are not used alone as a front line diagnostic test but are used in combination with pan-specific diagnostic approaches.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2016.08.002.

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